Generating Mouse Models for Studying the Function and Fate of Intrinsic Cardiac Adrenergic Cells

KARL PFEIFER,[a] STEVE P. BOE,[a] QI RONG,[b] AND STEVEN N. EBERT[b]

[a]*Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, Bethesda, Maryland 20892, USA*

[b]*Department of Pharmacology, Georgetown University Medical Center, Washington, District of Columbia 20007, USA*

ABSTRACT: Embryos lacking the ability to synthesize epinephrine and norepinephrine die (probably due to cardiac failure) without exogenous supplementation while mutant neonates can grow into fertile adults without supplementation. These experiments define a critical period during embryogenesis, when norepinephrine and/or epinephrine are essential for mouse development. The critical period is prior to sympathetic innervation of the heart and prior to synthesis of catecholamines by the adrenal medullae. Recent work indicates that the developing heart is likely to be a major source of catecholamines in the developing mammalian embryo. The spatial pattern of biosynthetic enzymes suggests an association of the intrinsic cardiac adrenergic cells with the developing pacemaker and cardiac conduction cells. To address the functional characteristics and the fate of these cardiac adrenergic cells, we have developed two mouse models that allow us to identify and to characterize the adrenergic cells and their descendants.

KEYWORDS: dopamine-beta-hydroxylase; phenylethanolamine N-methyltransferase; green fluorescent protein gene

INTRODUCTION

Cardiac function is profoundly affected by stimulation with epinephrine and norepinephrine. Both heart rate and the effectiveness of each beat are increased upon catecholamine stimulus. In adult mammals, the primary sources of the catecholamines are the sympathetic nervous system and the adrenal medullae where they are synthesized by the enzymatic conversion of L-tyrosine (FIG. 1).

The developing embryonic heart is also sensitive to catecholamine stimulation.[1–3] Genetic experiments from Richard Palmiter's lab suggest that the role of catecholamines is actually even more critical in the embryonic than in the postnatal heart.[4] Mice homozygous for a disruption of the *dopamine-beta-hydroxylase* (*DBH*) gene are unable to synthesize either epinephrine or norepinephrine and such mice die during

Address for correspondence: Karl Pfeifer, Building 6B, Room 206, 9000 Rockville Pike, Bethesda, MD 20892, USA. Voice: 301-402-0676; fax: 301-402-0543.
e-mail: pfeiferk@mail.nih.gov

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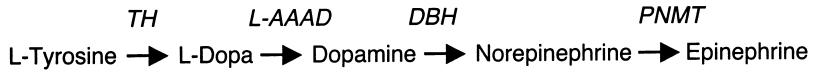

FIGURE 1. Catecholamine biosynthetic pathway. The catecholamines, norepinephrine, and epinephrine, are derived from L-tyrosine as described, with the enzymes catalyzing each reaction shown in italics. TH, tyrosine hydroxylase; L-AAAD, L-aromatic amino acid decarboxylase; DBH, dopamine β-hydroxylase; PNMT, phenylethanolamine N-methyltransferase.

embryogenesis beginning at e11.5. Although not definitively proven, the cause of death appears to be cardiac failure. Importantly, the application of exogenous catecholamines rescues the mutant embryos so that viable *DBH*−/− pups can be obtained with the expected Mendelian frequency. The requirement for exogenous catecholamines is strictly embryonic: mutant neonates do not require continued supplementation to develop normally. Thus, there is a critical period during embryogenesis when catecholamines are strictly required for cardiac function and/or normal development.

DISCUSSION

Catecholamine Synthesis in the Embryonic Mammalian Heart

The requirement for catecholamines during embryogenesis presented a developmental biology puzzle because the critical period is prior to both the sympathetic innervation of the heart and to the production of catecholamines by the adrenal gland.[5–7] Thus, the question arose as to the source of epinephrine and norepinephrine during this critical period. A number of recent studies have indicated that the embryonic heart itself is likely to be a major source of catecholamines.[7,8] This has now been demonstrated in chick, and in several mammals including rat, where both catecholamines and the RNAs encoding the biosynthetic enzymes have been identified.

In the rat, for example, mRNA encoding for the epinephrine biosynthetic enzyme, phenylethanolamine N-methyltransferase (PNMT), first appears on e9–9.5 (FIG. 2), which roughly corresponds to the early somite stage when the first myocardial contractions occur. As previously shown,[7] PNMT expression is restricted to the heart at these early embryonic stages of development. Upon examination of PNMT mRNA in the heart itself, we found that relatively high levels were maintained through gestational day 12 (e12), but then expression began to decline dramatically through much of the remaining portion of prenatal development (e13–e19) (FIG. 3). These results are consistent with the transient nature of catecholamine biosynthetic enzyme expression in developing rats,[7] and when considered in context of the embryonic lethality associated with catecholamine deficiency in mice, suggests that local production of catecholamines by the heart itself may serve a critical developmental function.

We wished to extend these findings by determining the spatial patterns of the intrinsic cardiac adrenergic (ICA) cells responsible for epinephrine and norepinephrine synthesis.[9] Using antibodies to the biosynthetic enzymes, PNMT, DBH, and TH, as well as to α-Actinin, we characterized expression patterns in the heart from e10.5 onward. Initially, cells expressing catecholamine biosynthetic enzymes were found interspersed throughout the developing heart, but as development proceeded

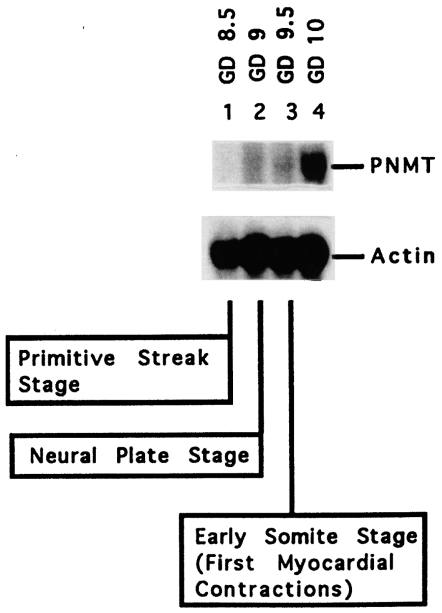

FIGURE 2. *PNMT* mRNA first appears during gestational day 9–9.5 in rat embryos. Total RNA was isolated from whole embryos on gestational days 8.5, 9.0, 9.5, and 10.0. Samples of 20 and 2 μg of total RNA were analyzed for *PNMT* and β-actin, respectively, using an RNase protection assay.[7] Developmental landmarks are designated below the appropriate lanes. GD, gestational day (equivalent to embryonic day, with 0.5 defined as noon of the day plugs were found).

they became transiently associated with specific areas of the heart. For example, at e11.5, the enzymes are expressed in the dorsal venous valve and the atrioventricular canal regions. These are respectively, the sites of the future sino-atrial (SA) and atrioventricular (AV) nodes. As development proceeds, expression is lost in these

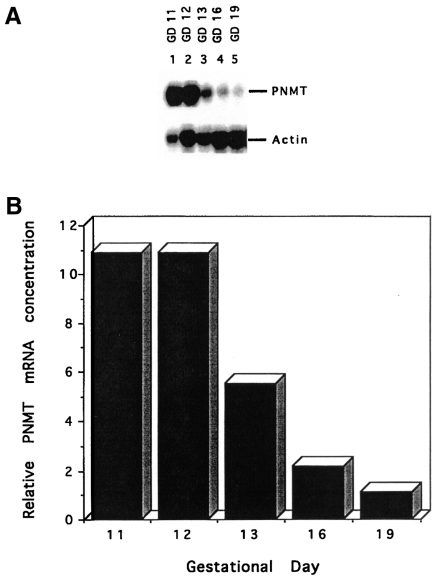

FIGURE 3. Temporal expression of *PNMT* mRNA in the prenatal rat heart. (**A**) Total RNA was isolated on the designated gestational days and analyzed by RNase protection assay. (**B**) Relative concentrations of *PNMT* mRNA in the embryonic/fetal heart as quantified by scanning densitometry of the film shown in (**A**).

TABLE 1. Summary of PNMT localization studies

Embryonic Stage	Primary Sites of Enzyme Distribution
E10.5	Diffuse staining: truncus arteriosis and primitive atrial and ventricular chambers
E11.5	Dorsal venous valve region and AV canal (SA and AV node regions)
E12.5–E13.5	AV junction (AV node)
E16.5	Crest of interventricular septum (early His bundle)

regions but noted in the interventricular septum. These expression patterns are summarized in TABLE 1. Altogether, the data demonstrated a transient and progressive association of the ICA cells with early cardiac pacemaking and conduction tissue development.

In sum, embryonic survival, in contrast to survival of postnatal mice, is absolutely dependent upon norepinephrine and/or epinephrine. The cause of death of the embryos appears to be cardiac failure. The embryonic heart appears be the source of the catecholamines, and the distribution of adrenergic cells within the developing heart is dynamic and suggests an association between ICA cells and the developing conduction system. Together, these results raise at least three interesting questions. First, what is the nature of the requirement for catecholamines by the embryonic heart? Second, what are the characteristics of the ICA cells? Third, what are the fates of the ICA cells? (Specifically, do ICA cells give rise to pacemaking and conduction cells?) We have taken a genetic approach to address these issues, and, specifically, we have generated two novel mouse genetic lines to isolate and characterize the ICA cells and to follow their fates in the developing heart.

Mutagenesis of the Mouse Pnmt Locus

The *Pnmt* gene encodes phenylethanolamine N-methyltransferase that catalyzes the conversion of norepinephrine to epinephrine. The mouse *Pnmt* locus is a relatively simple and compact gene consisting of only three exons (FIG. 4).[10] We are generating two distinct mutations at the locus by introducing insertions into exon 1 (FIG. 4). The *Pnmt::GFP* allele carries an insertion of the *Green fluorescent protein* (*GFP*) gene fused in-frame to the endogenous *Pnmt* ATG translation initiation codon. The *GFP* also carries a *Nuclear Localization Signal* (*NLS*) sequence to concentrate the GFP protein to the cell nucleus and thereby increase sensitivity of GFP detection. GFP is a naturally occurring fluorescent protein from *A. victoria*. Mice carrying this mutation are expected to synthesize GFP protein in any cell type that normally expresses the *Pnmt* gene. Thus all ICA cells, as well as adrenergic cells in other tissues, will be marked for localization and purification. Cells will be marked by the presence of GFP only as long as the cells remain adrenergic (ignoring any differences due to different half-lives of GFP and the catecholamine biosynthetic enzymes). The particular advantage of the GFP marker is in the ability to detect its fluorescence in living cells and the consequent ability to sort living cells based on *Pnmt* expression.

In addition to the *GFP* gene, a *NeoR* cassette flanked with FRT sites was introduced for positive selection, and the *Diphtheria Toxin A* (*DT-A*) gene was used for negative

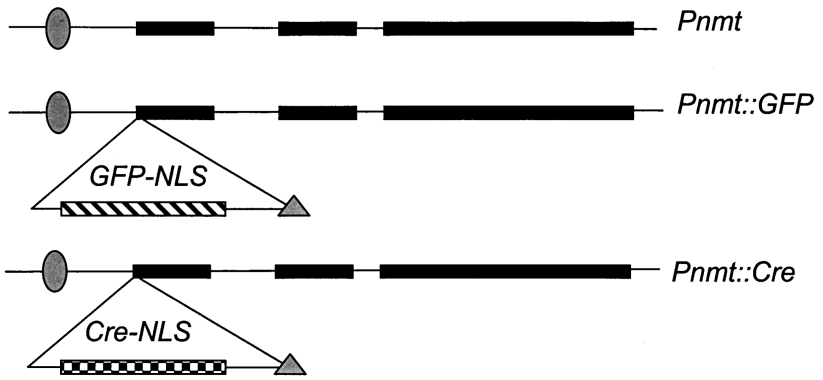

FIGURE 4. Wild-type and mutant *Pnmt* alleles. Cartoon depiction of the wild-type (*Pnmt*), *Pnmt::GFP*, and *Pnmt::Cre* alleles, indicating the *Pnmt* promoter (filled oval) and three exons (filled rectangles). *Pnmt::GFP* carries an insertion of sequences encoding the Green Fluorescent Protein while *Pnmt::Cre* carries an insertion of sequences encoding the Cre recombinase enzyme. In each case, homologous recombinants were selected positively by resistance to G418. However, the *NeoR* cassette was flanked with FRT sites allowing its removal via *Flp* recombinase. *NLS,* Nuclear localization signal.

selection of embryonic stem cells. G418 resistant clones have been screened for homologous recombination, and positive clones have been used to generate chimeric founder mice.

The *Pnmt::Cre* allele is similar but, instead of *GFP,* carries an in-frame insertion of the *Cre recombinase* coding sequences. We have generated and analyzed mice carrying this mutation with the hopes of undertaking two experiments. First, we expect that the insertion of the *Cre recombinase* gene will disrupt normal *Pnmt* gene function and prevent the conversion of norepinephrine to epinephrine. By analyzing mice homozygous for the insertion, we will thus be able to identify specific roles of epinephrine in mouse development and physiology.

The second use of the *Pnmt::Cre* allele is in identifying the fate of adrenergic cells, specifically of ICA cells. In this experimental system, we will make use of a tester strain developed by Philippe Soriano in which a modified *lacZ* gene was introduced into the constitutively active *Rosa26* locus.[11] Normally, insertion at this locus permits gene expression uniformly in all cell types. However, in this case, Soriano introduced a floxed "STOP" sequence at the 5′ end of the *lacZ* insertion. The presence of this STOP sequence prevents *lacZ* expression. The *loxP* sequences flanking the STOP are targets for site-specific recombinase activity by Cre enzyme whose action will thus result in their removal and the subsequent activation of the *lacZ* gene. Thus, in mice heterozygous for the *Pnmt::Cre* allele, cells that normally synthesize epinephrine will now also make Cre protein, which will result ultimately in a the cell becoming positive for β-galactosidase enzyme activity. Because the effect of cre synthesis is a genetic change (i.e., the excision of the STOP DNA sequences), adrenergic cells and their descendants are permanently marked by β-galactosidase. In contrast, the *Pnmt::GFP* allele will mark cells (as GFP positive) only as long as expression of the *Pnmt* persists.

SUMMARY

Through mutagenesis of the *Pnmt* locus, we are generating a mouse strain that allows us to identify, isolate, and characterize intrinsic cardiac adrenergic cells, and a second strain that allows us to identify, isolate, and characterize the descendants of these cells. Catecholamine biosynthesis is essential to normal mouse embryogenesis, and the developing heart appears to be the primary site of catecholamine synthesis. We hope that these mouse strains will allow us to identify the specific requirement for catecholamines in the developing embryo and to understand the nature of the cardiac cells that synthesize these hormones.

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